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TITLE: Early Detection Based on Angiogenic Growth Factors in Nipple Aspirate Fluid

PRINCIPAL INVESTIGATOR: Bruce J. Trock, Ph.D.

CONTRACTING ORGANIZATION: Georgetown University
Washington, DC 20057

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PREPARED FOR: U.S. Army Medical Research and Materiel Command
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13. ABSTRACT ***(Maximum 200 Words)***

The objective of this proposal is to develop new methods of early breast cancer detection by identifying increases in angiogenic growth factor secretion in nipple aspirate fluid (NAF). Specifically, the study is examining basic fibroblast growth factor (FGF-2) and vascular endothelial growth factor (VEGF), two of the most potent angiogenci molecules whose expression is thought to increase as an early event in breast carcinogenesis.

This project has been delayed repeatedly by unavoidable problems in staffing, collaborators leaving the institution, the PI's own move to a different institution, institutional IRB problems, and competition for patients. The net result is that, at the conclusion of the funding period, sufficient patient samples have not been accrued to complete the study goals. However, the PI has funding from the National Cancer Institute to study other potential biomarkers of early detection in NAF, specifically protein expression patterns. Samples from this study will be used to supplement those already obtained with DOD funding, and analyses for FGF-2 and VEGF will be performed using institutional funds available to the PI.

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FOREWORD

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NA In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

✓ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

NA In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

NA In conducting research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

NA In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Bruce Trock

PI – Signature

7/30/02

Date

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INTRODUCTION

The objective of this proposal is to develop new methods of early breast cancer detection by identifying increases in angiogenic growth factor secretion in nipple aspirate fluid (NAF). Specifically, the study is examining FGF-2 (basic fibroblast growth factor) and VEGF (vascular endothelial growth factor), two of the most potent angiogenic molecules whose expression is thought to increase as an early event in breast carcinogenesis. By comparing levels of these growth factors in NAF samples from women from three groups, i.e. those with normal breasts, DCIS (ductal carcinoma in situ), and early invasive breast cancer, we will determine whether increases in either of these molecules heralds the transition to the pre-invasive and/or invasive phenotype.

This project has been delayed repeatedly by unavoidable problems in staffing, collaborators leaving the institution, the PI's own move to a different institution, institutional IRB problems, and competition for patients. The net result is that, at the conclusion of the funding period, sufficient patient samples have not been accrued to complete the study goals. However, the PI has funding from the National Cancer Institute to study other potential biomarkers of early detection in NAF, specifically protein expression patterns determined by surface enhanced laser desorption and ionization spectroscopy (SELDI). Samples from this study will be used to supplement those already obtained with DOD funding, and analyses for FGF-2 and VEGF will be performed using institutional funds available to the PI. This will allow not only the completion of the original study goals to examine the two angiogenic biomarkers, but will also allow us to identify other associations between these biomarkers and protein expression profiles in NAF. Thus, although the study will not have been completed in the original timeframe, the possibility to gain increased understanding of the mechanisms of angiogenesis in breast neoplasia will add to the value of the research originally proposed.

BODY

Progress and problems during the funding period will be described below with respect to each of the tasks in the original Statement of Work. Before describing each task however, an update on the institutional affiliation of the PI (Dr. Trock) is necessary.

In April 2001, Dr. Trock left Georgetown University, Lombardi Cancer Center to assume a position at the Johns Hopkins School of Medicine, Departments of Urology, Oncology and Epidemiology. The original funding period of the grant was scheduled to expire on June 30, 2002. The balance of funds on the grant remained at Geogetown University to cover the salaries of project staff who remained at Georgetown, to continue working on project-related tasks until the end of the funding period. No funds from this grant were transferred to Johns Hopkins.

Note: Tasks 1 and 3 will be discussed together because the problems to be discussed affected both activities.

Task 1: Coordinate with physicians, nurses and scheduling secretaries to receive schedules of patients who will undergo breast surgery (Surgery Clinic), and patients who will attend the Comprehensive Breast Center, or the Breast Cancer Consultation Group.

Task 3: Implement patient accrual and NAF collection (Months 2-30).

To date, samples have been obtained from 128 women. There were a number of problems that significantly delayed progress in this area. During the first year of the study there was considerable turnover among ancillary staff at the three Georgetown clinics where patients were recruited. This made it difficult for the Research Assistant who enrolled patients to be informed of eligible patients in a timely fashion, and reduced the time that physicians had available to introduce the study to patients and determine their interest in participating. It also resulted in enrollment of many patients who were not truly informative for the study aims, i.e. patients who had previously had surgery for breast cancer and were being seen in the clinic for routine follow-up. Thus, the sample would not reflect tumor-associated growth factor levels. These patients only contributed NAF from the contralateral breast. Although these patients are of interest (i.e. potential early detection of 2nd primaries), they are not the primary target population for the study. Similarly, a number of women were enrolled in the clinic soon after their biopsy, so their affected breast (i.e. presumed to have tumor) was too sore to permit the NAF procedure. These women also contributed NAF from the contralateral breast, which, while of potential interest, does not allow us to address the early detection goals of the study. To surmount these problems, we arranged for the Research Assistant to be present in the clinics during the times when eligible patients were scheduled. This improved the rate of enrollment. The primary sources of patients

were the Breast Surgery clinic at Georgetown and a former Georgetown surgeon who maintained a private practice in breast surgery. Enrollment was proceeding at approximately 2-4 patients per week.

In April 2001, Dr. Trock moved to Johns Hopkins, as described above. The intent was to continue activities until June 30, 2001 (the end of the original funding period), and then begin recruiting patients from the breast surgery practice at Johns Hopkins University (JHU). However, shortly after arriving at JHU, all research activities at the medical center were suspended in July 2001 by the Office for Human Research Protection (OHRP), due to the death of a research subject (this was entirely unrelated to Dr. Trock's research). This suspension was brief, but resulted in all new IRB applications being put on hold. This delayed submission of the IRB application to begin this study at JHU. Subsequently (October 2001), JHU arranged for an outside IRB, Western Institutional Review Board (WIRB) to review new applications. We submitted our protocol to the WIRB and it was approved Jan 25, 2002 (WIRB protocol # 20020089) (consent form, protocol, and WIRB approval letter included as **Appendices 1-3**). This protocol was to allow us to enroll patients and collect NAF to be analyzed for the angiogenic growth factors that are the subject of the current project, and for analysis of protein expression patterns via SELDI that are the subject of a grant funded by the National Cancer Institute (Detecting Breast Cancer Protein Signatures in Body Fluids, Grant No. CA85082-03).

Additional delay was caused by difficulties in setting up a system to enroll newly diagnosed breast cancer patients and controls. The primary breast cancer surgeon at JHU left in the fall of 2001, and was not replaced until April 2002. Because there were already two other investigators with previous interests in enrolling from the same patient population, there was a need to work out arrangements for sharing access to patients. To avoid overburdening patients with multiple investigators trying to enroll the same patients, Dr. Trock and the other investigators worked out a protocol where patients can initially be offered the opportunity to enroll in the NAF study or the studies of the other investigators (involving breast ductal lavage). For patients who agree to the ductal lavage, we will first obtain a NAF sample (since this is part of the NAF protocol anyway) prior to conducting the lavage. Developing the protocols to conduct these overlapping but not completely similar studies has been difficult because Dr. Trock and the other investigators had to agree on enrollment and data/sample collection procedures that were appropriate for all studies, and the need to share samples and data among multiple investigators. We also had to develop complementary questionnaires, protocols and consent forms, and recruitment scripts. These activities required us to submit amendments to our WIRB protocol (which have been approved).

Finally, we had planned to enroll patients through the Breast Imaging Clinic. Potential cases are those recommended for breast biopsy, and controls are those whose mammogam was normal. However, the chief of the Breast Imaging Clinic was planning to take a position elsewhere, so

she did not want to begin any new protocols. She referred us to the interim chief. He has agreed to allow us to enroll patients, and we have implemented a protocol with his research assistant for identifying patients and asking them if they would like to discuss the study. We hope that subjects approached at this time will be more willing to enroll; they will be called several days after they have recommended for biopsy, and asked to come in for a visit prior to the date of the biopsy. In this way, they will have had time to cope with the impending biopsy, rather than being approached in the clinic on the day that they learn that a biopsy is necessary. In addition, because we could not count on having an exam room available in the Breast Imaging Clinic to allow the NAF procedure and patient interview to be conducted with privacy, we applied for and received approval (June 13, 2002) to conduct the procedures in the JHU General Clinical Research Center (GCRC).

The upshot of all of these factors is that we have not enrolled patients since April 2001. However, we have begun asking patients in the Breast Imaging Clinic if they would be interested in learning of the study and possibly participating. Those who indicate that they are interested are called by the Research Assistant, and the study is explained to them. If they are interested in participating, they are scheduled for a date to come to the GCRC prior to their breast biopsy, when they provide informed consent and have the procedure performed.

We will begin enrolling patients in the breast surgery clinic at the end of August, when the breast surgeon returns from vacation. At that time we will assess the rate of enrollment from surgery and the Breast Imaging clinic. If it appears that we will enroll less than 4 patients per week, we will re-activate the protocol at Georgetown University, with our former collaborator at Georgetown (breast surgeon Dr. Marie Pennanen) as the PI. We need to enroll approximately 80 additional subjects. This should be completed in one year, including laboratory assays.

Task 2: Develop appropriate quality control methods for VEGF and FGF-2 assays.

This task was completed in the first year of the study and reported in the first Annual Report. A feasibility issue concerned the need to process and freeze the samples within a short time of collection. Since the research assistant sometimes remained in the clinic the entire day, operations could be made more efficient if processing and freezing could be delayed until the end of the day. Serum samples spiked with increasing concentration of FGF-2 were tested under control conditions and with addition of two different protease inhibitors (aprotinin 2 μg/ml, and phenymethyl sulfonyl fluoride 100 μM), to determine whether the protease inhibitors could prevent degradation of FGF-2 at room temperature. These studies showed that the protease inhibitors were not effective in preventing the degradation if samples were left at room temperature overnight. This required us to set up a system to notify the lab for rapid pickup of the samples from the clinic

Task 4: Conduct ELISA assays for VEGF and FGF-2 (Months 4-30).

Dr. Sandra McLeskey, the Co-PI who was overseeing the assays in her lab, left Georgetown after the first year to join another university. Dr. Dorraya El-Ashry, a breast cancer basic scientist at Georgetown assumed responsibility for the laboratory assays in her lab. She performed VEGF analyses on 48 samples from 33 patients (for 15 patients, samples from both breasts were analyzed). In addition she analysed samples of breast cyst fluid from an additional 11 patients. These patients were included to provide us with a wider range of breast abnormalities that may influence composition of breast secretions. The data from these assays were included in the second Annual Report and demonstrated a wide range of expression (< 500 – 139,000 pg/ml). For some subjects expression levels from the left vs. right breasts demonstrated large differences (e.g. 26000 vs. 73200 pg/ml). In the fall of 2001, Dr. El-Ashry left Georgetown to go to the University of Michigan. We have arranged to have the ELISA assays performed by the Clinical Chemistry Laboratory in the Department of Pathology, under the auspices of Dr. Daniel Chan, who has considerable experience with ELISA (Wang Y, 2001). Dr. Trock will pay for these analyses using institutional research funds. These assays should be completed in one year.

CONCLUSION

This study has been beset by an unusually large number of difficulties that have limited accrual to the study and analysis of collected samples. These problems include turnover in clinic staff and co-investigators/collaborators, need to approach the patient at a time of great anxiety, the PI's move to another institution, suspension of all human subjects research at the new institution and resultant delays in the IRB approval process, and competition for the target patient population at the new institution. Despite all of these problems and the delays in progress to date, we still plan to complete this study. Dr. Trock is the PI of an NCI-funded study of NAF for early detection. This study targets the same patient population and has the same early detection goals as the current study; only the candidate biomarker is different. Because the SELDI assays for protein expression for that study require only a portion of the NAF sample from any given patient, we will be able to also assay the samples collected for that study for FGF-2 and VEGF.

We anticipate that recruitment of women recommended for biopsy in the Breast Imaging clinic, as well as women in the Breast Surgery clinic will permit us to complete recruitment within one year (this would require only 1-2 subjects per week). Dr. Trock will use institutional research funds available to him to cover the additional costs (i.e. purchase of additional ELISA kits for FGF-2 and VEGF, and technician time). The IRB-approved consent form and protocol for this study at Johns Hopkins University were submitted to the IRB after the end of the funding period

for the current grant had expired, and therefore were not submitted to the DOD. Although these documents bear the title of the NCI-funded grant ("Detecting Breast Cancer Protein Signatures in Body Fluids"), the consent form and protocol specifically mention analysis of the samples for growth factors as well as protein expression (consent form: page 1, last paragraph; protocol: page 6, last paragraph). **The Consent Form, Protocol, and IRB Approval letter are included as Appendices 1-3.**

REFERENCES

Wang Y, Kuhajda FP, Sokoll LJ, Chan DW. Two-site ELISA for the quantitative determination of fatty acid synthase. Clin Chim Acta 2001;304:107-15.

APPENDICES

1. Current IRB-approved consent form at Johns Hopkins University (pps. 12-18)
2. Current IRB-approved protocol at Johns Hopkins University (pps. 19-31)
3. Letter of approval from the Western Institutional Review Board (pps. 32-33: this IRB supplemented the JHU IRB to handle the backlog of applications after the suspension of human subjects research at JHU was lifted)
4. Approval from the Johns Hopkins University General Clinical Research Center (pps. 34-35)
5. List of abbreviations and acronyms (p. 36)
6. Meeting abstracts during reporting period (p. 37).
7. Publications during reporting period (p. 37).
8. Manuscripts in preparation (p. 37).
9. Personnel receiving pay from this negotiated effort (p. 37).

The Johns Hopkins Medical Institutions
(The Johns Hopkins Hospital
The Johns Hopkins Bayview Medical Center, etc.)

Subject I.D. Plate

APPROVED
Jan 25, 2002
WIRB®
Olympia, WA

RESEARCH SUBJECT INFORMATION AND CONSENT FORM

Title: Detecting Breast Cancer Protein Signatures in Body Fluids

Protocol No.: U01-CA850082-01
WIRB® 20020089

Sponsor: National Cancer Institute
Bethesda, MD

Principal Investigator: Bruce Trock, Ph.D.
The Johns Hopkins University Medical Center
Departments of Urology and Epidemiology
600 N. Wolfe Street
Jefferson Street Building, Room 149
Baltimore, MD 21287
(410) 502-2773
(410) 955-4494 (24 hours)

Sub-Investigators: Kathy Helzlsouer, M.D., M.H.S. Telephone: (410) 955-9727
Marie Pennanen, M.D., Telephone: (202) 687-8595
(Georgetown University)

This consent form explains the research study and may contain words that you do not understand. Please read it carefully. Ask questions about anything you do not understand. If you do not have questions now, you may ask later. You may take home an unsigned copy of this consent form to think about or discuss with family or friends before making your decision.

Purpose:

You are being asked to give permission to Johns Hopkins Medical Institutions to collect biological samples such as blood or nipple aspirate fluid (called "NAF" for short). NAF is secreted naturally by a woman's breast, and is obtained non-invasively. This means it does not involve needles or any cutting. These specimens will be used in research to study whether women with increased risk of breast cancer or those with very early breast cancer can be detected by measuring biological indicators or "biomarkers" in these fluids. These biomarkers may include protein patterns and specific growth factors. This research study will enroll women coming to clinics at Johns Hopkins University Medical Center, and other clinics in the Washington, DC metropolitan area. Approximately 240 women will participate in this study.

The general plan of the research study:

You will be asked to fill out a questionnaire asking for information such as your medical history, family history of cancer, and possible risk factors for breast cancer. You may fill out the questionnaire at the clinic, or a self-addressed stamped envelope will be given to you so that you can complete it at home and return it to Dr. Trock at your convenience.

NAF or blood specimens will be obtained as described below. If you are having a breast biopsy or surgery, the study doctor will ask you if we may store some of the tissue that is left over and would ordinarily be discarded. This tissue sample will not affect any decisions about your treatment. You will not have a separate procedure to collect this tissue. You may decide to provide only one of the samples, such as NAF. **Whether or not you agree to participate in this study, your decision will not affect the other tests or medical care that your physician has recommended for you at this site.**

The following will take place if you participate in this research:

NAF specimens: A specially-trained breast research assistant will describe the procedure and study to you and answer any questions that you may have. Before the procedure the research assistant will have you stroke your breast in a downward motion to help move the fluid inside your breast toward your nipple. The research assistant may also place a warm compress on your breast for several minutes. Your breast will be cleaned with alcohol, and the research assistant will place a small, smooth plastic or glass cup over your nipple. The other end of the cup is connected to a syringe (no needles are involved). Suction is applied to the nipple by pulling back the plunger in the syringe. You will need to gently squeeze your breast with both hands near the base of the breast. The research assistant will apply pressure from the pump for 8 to 10 seconds.
NAF is not always obtained at first try. Several attempts may be made to obtain NAF; each attempt will be like the first and last 8 to 10 seconds. The research assistant will use the pump to obtain NAF from each breast. Any NAF that appears on your nipple will be collected in a thin tube.

Blood specimens: A needle will be placed into one of the veins in your hand or arm. Approximately four to six tablespoons of blood will be taken, using standard blood collection techniques. From these blood samples, the serum and plasma (liquid portion of your blood) and lymphocytes (white blood cells) will be stored. Remaining serum, plasma and lymphocyte samples may be used in future research on breast cancer after the current study ends.
Risks:

The suction pressure from the pump may be uncomfortable, but this feeling should stop when the pump is stopped. You should tell the research assistant immediately if it becomes too uncomfortable for you. There is a chance that a bruise or minor scratching may develop where the pump is placed on the breast. If you are going to have a biopsy or

surgery on your breast, the procedure for obtaining NAF will not affect the results of your biopsy or surgery, or the on the healing process.

If a blood sample is obtained, mild pain or bruising, lightheadedness or infection may result from the needle stick. If you are having a biopsy or surgery there are not additional risks associated with collection of breast tissue beyond those associated with the surgical procedure itself, which has been explained to you by your doctor and ordered as part of your care.
There may be risks or side effects that are unknown at this time.

Benefits:

Although you will not directly benefit from providing these specimens, future generations may benefit from increased medical knowledge about breast cancer. Because these samples will be used for research purposes only, you will not receive specific information on the results of any research test performed on your samples.

Costs:

There are no costs to you for providing NAF blood samples. You or your insurance company is responsible for costs related to a biopsy or surgery.

Providing the tissue will not result in additional charge.

Payment for Participation:

You will not be paid for providing specimens.

Alternatives:

This is not a treatment study. Your alternative is not to participate in this study.

Compensation for injury:

It is not possible to predict everything that might occur. If you are injured as a result of being in the study, or thing you have not been treated fairly contact Dr. Trock at (410) 502-2773. The services at the Johns Hopkins Hospital or the Johns Hopkins Bayview Medical Center will be open to you in case of any such injury. However, the Johns Hopkins University, the Johns Hopkins Hospital, the Johns Hopkins Bayview Medical Center and the federal government do not have a program to pay you if you are hurt or have other bad results associated with participating in this study.

You and your insurance company will be responsible for payment of any treatment or hospitalization you require if you are injured. It is up to you to check with your insurance company before you start this study to find out what your insurance company would pay for. Additional information about this may be obtained from the Joint Committee on Clinical Investigation at (410) 955-3008.

Source for funding:

Funding for this research study will be provided by the National Cancer Institute.

Commercial Issues:

There are no plans to compensate you for any products developed from the use of your samples in this research study.

Confidentiality:

All information and samples collected from you will be identified with a code number to prevent unintentional disclosure of information that could identify you. Your records and biological samples will be kept a minimum of five years or until your samples are used up. If you request to be dropped from the study before this time, then all your information and samples will be destroyed. State law requires us to report certain contagious diseases or if we find information about child abuse.

Information from this study may be given to the sponsor, the National Cancer Institute. "Sponsor" also includes any persons or companies that are contracted by the sponsor to have access to the research information during and after the study.

The information may also be given to the U. S. Food and Drug Administration (FDA). It may be given to governmental agencies in other countries. Medical records which identify you and the consent form signed by you will be looked at and/or copied for research or regulatory purposes by:

- The sponsor;

And may be looked at and/or copied for research or regulatory purposes by:

- The FDA
- Department of Health and Human Services (DHHS) agencies;
- National Institutes of Health (NIH);
- Governmental agencies in other countries;
- Johns Hopkins University; and
- Western Institutional Review Board® (WIRB®).

Absolute confidentiality cannot be guaranteed because of the need to give information to these parties. The results of this research study may be presented at meetings or in publications. You will not be identified in any publish research report.

Questions:

If you have questions concerning your participation in this study or if at any time you feel you have experienced a research-related injury, contact the study doctor:

Dr. Trock at (410) 502-2773
(410) 955-4494 after hours.

If you have any questions about your rights as a subject in a research study, you may contact:

Western Institutional Review Board® (WIRB®)
3535 Seventh Avenue, SW
Olympia, Washington 98502
Telephone: 1-800-562-4789

WIRB is a group of people who perform independent review of research.

Do not sign this consent form unless you have had a chance to ask questions and have received satisfactory answers to all of your questions.

New findings:

During the study, you will be told any new facts that might change your decision to be in the study.

Voluntary participation/Withdrawal:

Participation in this study is voluntary. You do not have to join this research study. If you do join, and later change your mind, you may quit at anytime. If you refuse to join the study, you will not penalized or lose any benefits to which you are otherwise entitled at this institution.
Your participation in this study may be stopped at any time by the study doctor or the sponsor without your consent.

Making Your Choice

Please read each sentence below and think about your choice. After reading each sentence, write you initials to the right of "Yes" or "No." **No matter what you decide to do, it will not affect your care at this institution.** If you have any questions, talk to your study doctor or study nurse, or call Dr. Trock at (410) 502 –2773.

1. My nipple aspirate fluid may be obtained for use in research related to breast cancer.

YES____(Initials) **NO____(Initials)**

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2. A sample of my blood may be obtained for use in research related to breast cancer.

YES____(Initials) **NO____(Initials)**

---

3. (If you are having biopsy or surgery) My breast tissue specimen may be obtained for use in research related to breast cancer.

YES____(Initials) **NO____(Initials)**

---

4. I wish to be contacted before my samples can be used for any future tests not discussed in this consent form.

YES____(Initials) **NO____(Initials)**

Consent:

I have read and I understand the information in this consent form. Anything I did not understand was explained to me. I have had all of my questions answered to my satisfaction. I understand that I may ask additional questions at any time. I freely consent to participate in this study.
I understand that I will receive a signed and dated copy of this consent form.

I authorize the release of my medical records for research or regulatory purposes to the sponsor, the FDA, the DHHS agencies, NIH, governmental agencies in other countries, Johns Hopkins University, and WIRB®.

By signing this consent form, I have not waives any of the legal rights which I otherwise would have as a subject in a research study.

_____
Printed Name of Subject

_____
Subject's signature Date

_____
Signature of Person Conducting Informed Consent Discussion Date

_____
Signature of Investigator (if different from above) Date

_____
Witness to Consent Procedures (Optional) Date

NOTE: A copy of the signed and dated consent form must be kept by the study doctor and a copy of the consent form must be placed in the subject's record.

Wirb/nci/20020089/1-25-2002/mf/tmb

APPLICATION FOR A NEW HUMAN SUBJECTS RESEARCH PROJECT

Title of project: Detecting Breast Cancer Protein Signatures in Body Fluids

Principal Investigator: Bruce J. Trock, Ph.D. Department: Urology

Title of Investigator: Associate Professor of Urology; Director, Division of Epidemiology

Signature of Investigator: Date of Signature:

Mailing Address: Brady Urological Institute
Johns Hopkins School of Medicine
600 N. Wolfe St.
149 Jefferson Bldg.
Baltimore, MD 21287

Telephone: 410-502-2773 Fax: 410-614-8096 Email: btrock@jhmi.edu

Co-Investigators: Kathy Helzlsouer, M.D., M.H.S.
Marie Pennanen, M.D. (Investigator at Georgetown Univ. site)

Study Location: Johns Hopkins Hospital, Georgetown University Department of Surgery

Funding/Sponsor: National Cancer Institute
Grant Number: U01-CA85082-01
Start Date of Grant: 10/1/99

ANSWER ALL OF THE FOLLOWING QUESTIONS:

Will marketed drugs or diagnostic reagents be administered? **NO**
IF YES, append a copy of the sponsor's protocol, if any.

Will investigational new drug(s) be administered? **NO**
IF YES, *supply the following information:*
DRUG NAME(s): N/A IND #:_____
held by: Sponsor ___ Investigator ___

Will medical devices be used in the study? **NO**
IF YES, *identify below type of device:*
___ DEVICE NAME(s): _____N/A_____IDE #: _____
held by: Sponsor ____ Investigator ____

Will Clinical Imaging Services be utilized? **NO**
IF YES, *identify type :*
___ Ionizing radiation - Include an RCU 5 form
___ Ultrasound or other imaging tests?
___ JHH Radiology will provide the Imaging?

Will samples be tested in a laboratory/facility that does not have CLIA certification? **NO**

IF YES, provide information below, on whether the results will be given to the patient/subject, patient's primary physician or used for patient diagnosis or management.

Will any infectious or biohazardous agents or specimens be obtained? **YES Biosafety Division has been contacted, and a registration form has been sent.**

IF YES, *contact the Biosafety Division, Health, Safety & Environment (410-955-5918).*

Do any of the participating faculty (*or their immediate family, staff, or students*) have a financial *interest (royalty, equity, or consulting*) in the sponsor and/or products used in this project? **NO**

IF YES, *submit a written statement of disclosure to the designated official for review of conflict of interest at the investigator's institution of primary appointment.*

Will one of the Federally Funded Hopkins General Clinical Research Centers be utilized? **NO**

IF YES, *identify center(s) below*:

___ JHH Adult inpatient unit ___ JHH Pediatric outpatient unit
___ JHH Adult outpatient unit ___ JHH Pediatric inpatient unit
___ General Clinical Research Center at JHBMC
__ KKI GCRC

PRIMARY SUBJECT POPULATION(s) TO BE ENROLLED

Age range: **18 and older**

___ Males _X_ Females ___ Children _X_ Adults
___ Inpatients _X_ Outpatients ___ Non-patient volunteers ___ Employees
___ Staff (*JHH or JHBMC*) ___ Students
___ Subjects with Mental Disorders ___ Handicapped
___ Prisoners ___ Pregnant Women
___ Fetuses (*or fetal tissue*) ___ Cognitively impaired subjects

INCLUSION AND EXCLUSION CRITERIA

Inclusion:

(1) Subjects must be able to speak, read and write in English. This is to insure that the subject is truly able to give informed consent, and is able to complete the study questionnaire. The research grant award did not provide sufficient funds to hire translators, and, if a bilingual friend or relative accompanied the subject, we would have no way to verify that the nature of participation was being adequately explained to the subject.

Exclusion:

(1) Subjects with any mental impairment that would hinder the ability to provide informed consent.

(2) Subjects with previous cancer other than non-melanoma skin cancer. Other cancers may be associated causally with circulating factors, or may secrete circulating factors that could confound associations sought for breast cancer.

(3) Subjects with previous chemotherapy treatment. Such treatments often may impact on secretory mechanisms.

(4) Subjects with previous breast augmentation or reduction surgery. Such surgery may cut the breast ducts preventing the aspiration of breast fluid through the nipple.

SUBJECT ENROLLMENT

Number of subjects to be enrolled at the Hopkins site(s):	First year: 20	Total study: 80
Total number of subjects to be enrolled at all sites:	First Year: 30	Total study: 120

CONSENT DOCUMENTATION *(Identify Type of Consent)*

X A copy of the proposed consent form to be signed by all subjects is attached to the application.

BACKGROUND

History of current study. This study and the grant which funds it were initiated while Dr. Trock was a faculty member at Lombardi Cancer Center at Georgetown University Medical Center (GUMC). The study was approved by the GUMC IRB under a long-standing protocol of Dr. Trock's for investigation of a number of biomarkers in nipple aspirate fluid (IRB #94-039). Dr. Pennanen, a breast surgeon at GUMC, was the primary clinical co-investigator on the study. Dr. Trock allowed the protocol to be de-activated at Georgetown when he left in April 2001 to take a position at Johns Hopkins University (JHU). The grant was transferred to JHU after Dr. Trock relocated.

Problems with Current Early Detection Approaches. Mammographic screening for breast cancer is currently the best available approach for early detection in the general population. However, additional approaches are needed. Current mammography is associated with a sensitivity of 75% - 90% (1), but the positive predictive value is low, approximately 25% (2). Furthermore, although resolution continues to improve, mammography is still dependent on the existence of a mass lesion. Because many breast tumors will already have metastasized by the time a mass is detectable (3), a significant portion of mammographically detected tumors in women undergoing regular screening will already be disseminated and incurable. Furthermore, for the following reasons, mammography alone may not be sufficient for early detection in premenopausal women, particularly for young women with inherited susceptibility or other high risk profiles: (a) effectiveness of mammography has not been established in women younger than 40, and is controversial in women aged 40-49 (b) younger women have more dense breast tissue,

which reduces mammographic sensitivity, (c) tumor growth rates may be higher in younger women, and (d) women carriers of some germ-line mutations such as ataxia telangiectasia and possibly BRCA1/2 may have increased sensitivity to radiation and conceivably could be harmed by frequent mammograms (4-6). The need for improved methods of surveillance is especially critical in younger women. More widespread testing of young women for germline predisposing mutations (such as BRCA1/2) will soon result in thousands of young women at high risk for breast cancer, for whom conventional screening approaches may be inadequate. Thus, there is an urgent need for additional methods of early detection that can provide an adjunct to mammography.

Rationale for Basing Early Detection on Protein Expression Profiles. Early detection of breast cancer generally depends on finding a palpable abnormality during breast exam or a radiographic abnormality during breast imaging. Although these abnormalities may result in identification of a tumor whose pathologic stage may be considered "early", they represent relatively late events in the process of neoplasia. Palpable or radiographic lesions may be considered as the outcomes of a constellation of phenotypic events including alterations in signal transduction, cell-to-cell communication, cell cycle control, proliferation, angiogenesis, and motility. Each of these phenotypes, in turn, represents the accumulation of a number of genetic defects resulting in abnormal protein expression and/or phosphorylation. Thus, a cardinal feature of cancer should be a profile of numerous altered proteins which, in concert, confer (or are associated with) the invasive phenotype at a point in time prior to the development of a detectable mass lesion.

Most molecularly-based approaches that have been suggested or are under investigation for early detection are targeted at specific defects, such as oncogenes, tumor suppressor genes, growth factors, tumor antigens, or other gene products. The inherent problem is that none of these factors are present in a large majority of breast cancers, and some are not specific to cancer or to breast tissue, so the sensitivity and specificity of such approaches is low. Rather than targeting a specific abnormality that may only be present in a small subgroup of patients, we propose to identify *general patterns of protein expression* that distinguish the invasive phenotype from pre-invasive or normal tissue. Because of the large number of proteins whose expression is altered during neoplastic progression, it is highly likely that protein patterns that are specific to breast cancer can be discerned. Previously, the study of protein expression patterns from cells has been confined mostly to the separation and analysis by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), which has been used extensively to study changes in protein expression in cell lines and bulk tissue specimens (7-10). 2D-PAGE analysis, although a powerful tool for the analysis of complex protein mixtures, is a laborious time-intensive process. This technique has limitations on its ability to analyze very small proteins (<7000 Daltons), very basic proteins, and cannot detect low or moderately expressed proteins when analyzing lysates generated from only a few hundreds of cells (11). The approach that we will use, Surface Enhanced Laser Desorption Ionization Spectrometry (SELDI) works extremely well in detecting such proteins, allowing study of a much wider range of protein expression.

SELDI is a novel, highly sensitive and rapid methodology for the capture, characterization, and analysis of complex protein mixtures (12-14). SELDI has the theoretical protein detection sensitivity in the femtomole range and generates protein profiles from complex protein mixtures in as little as 5 minutes (12-14). This rapid analysis capability opens up the possibility of creating diagnostic expression profiles from tissue and body fluids which can help guide therapeutic and clinical decision making. SELDI allows for desorption and detection of intact proteins from crude samples (i.e. the intact tissue or body fluid environment) with the advantage that no purification or chemical modification of the sample is required for analysis. Using SELDI Protein Chip™ systems and arrays (Ciphergen Biosystems, Inc.),

tissue or body fluids containing proteins of interest are directly applied to distinct "spots" on the chip; each chip contains 8 to 24 predetermined spots. The chip is coated with defined chemical "bait" molecules that bind subsets of proteins based on their charge to mass ratios. Different chips are available that use different types of bait, allowing detection of a wide range of proteins (14). The bound proteins can then be washed with a variety of washing buffers so that only those proteins that share common chemical properties are retained on the surface.

Studies of Nipple Aspirate Fluid and Serum. An alternative to imaging technologies for breast cancer detection is to examine easily accessible biological fluids for evidence of molecular signatures associated with neoplastic change. Ideally such an approach should reflect the relevant biology of breast epithelium, be relatively non-invasive, easy for the patient, conducive to serial monitoring at intervals suitable for the risk status of a given woman. Nipple aspirate fluid and serum both provide feasible approaches.

Nipple aspirate fluid (NAF) may be the most promising of the biological fluids available because it provides, in a non-invasive fashion, a direct sampling of breast epithelial biology. NAF has been studied for many years as a means of identifying women with high risk of breast cancer or preclinical disease. This fluid is secreted continuously by the non-lactating breast and can be aspirated through duct openings in the nipple using a simple non-invasive pump. NAF is of interest because it has a relatively long retention time in the breast alveolar-ductal system, where it accumulates secreted factors and exfoliated mammary epithelial cells. *Thus, examination of NAF provides a "snapshot" of the micro-environment where breast cancer originates*. NAF can be sampled easily and inexpensively at regular intervals, providing a non-invasive means of monitoring biological changes in breast epithelium, and can be obtained from over 50% of healthy premenopausal women (15).

The contents of NAF are likely to be representative of the environment of breast ductal structures. Although the terminal ductal lobular units where cancer originates are usually not in close anatomic proximity to duct openings, the ducts provide anatomic continuity from the lobular units to the nipple duct openings. The fact that premalignant and malignant cells are found in NAF indicates that the physical distance from lobular units to duct openings does not prevent relevant biological components from being captured in NAF samples.

To date, classical cytologic assessment has been used to identify abnormalities in NAF-derived cells as an indicator of early progression toward breast cancer. However, this approach has low specificity. Although the presence of cytologic atypia in NAF-derived cells increases the risk for subsequent breast cancer development, relatively few women with cytologic atypia go on to develop breast cancer. Furthermore, there are no morphologic characteristics to distinguish those women with atypia who will progress to breast cancer from those who won't (16). It is likely, however, that epithelial cells destined to progress to cancer will have accumulated a number of premalignant or preinvasive molecular changes resulting in altered protein expression. Because of the large number of proteins that are altered in neoplasia, proteins whose structure is presently unknown may also be differentially expressed in normal and tumor tissue. *The approach that we are proposing depends on relative protein expression levels as indicated by a standard chromatographic molecular weight map, so differences in intensity of known as well as unknown proteins can be captured.* It is important to note that the approach we are proposing does not depend on the small number of epithelial cells typically present in NAF samples. Detection of proteins in *tumor tissue* requires generation of a cellular lysate to release protein contents of cells. However, NAF already contains secreted or shed proteins, in a volume larger than that produced by a lysate from a small tissue sample, so the cellularity of the NAF sample is not a limitation in the use of SELDI.

Serum Markers. A shortcoming of using NAF as a sampling medium is that the likelihood of obtaining fluid from an individual woman decreases with age. Prior to menopause, fluid can reliably be obtained from 50-75% of women (17); in our hands, the yield has been toward the higher end of this range. After menopause, this yield rate decreases rapidly. Therefore, serum is a potential alternative to NAF for postmenopausal women. Detection of circulating tumor markers in serum has been attempted for many years. Most approaches have focused on mucin-like high molecular weight (> 45,000 Daltons) cell membrane glycoproteins that are products of or related to the MUC-1 gene CA15-3, CA549, MSA) and circulating antibody responses against putative tumor antigens (18). However, there are a number of problems with these traditional serum markers. These markers tend to be detectable only with large tumor burden because they undergo rapid catabolic clearance from the circulation (19). Thus, they are unlikely to permit detection at very early stages of progression. Furthermore, they are neither specific to cancer nor to breast tissue, so even if detected, the origin of a cancer must be sought with traditional diagnostic techniques (18). Finally, detection has depended on development of monoclonal antibodies (mabs) that recognize specific epitopes on these molecules. However, different epitopes can have large differences in their value as a targeting mechanism for serum detection. These differences can be due to variation in glycosylation patterns, polymorphic mRNA splice variants resulting in different circulating forms, and different functional domains of the molecule (20).

We propose to use SELDI to assess protein expression patterns in serum. An additional advantage of this approach for serum relates to the range of proteins that can be detected. Traditional serum markers are detectable because they are readily shed from the cell surface. However, intracellular proteins may be more relevant because they reflect processes of signal transduction and other critical biological processes altered in neoplasia. Many of these proteins are small enough (<10,000 Daltons) to pass through cell membranes into circulation. Antibody-based methods may not work well for such small proteins, especially if they are not highly expressed, and require you to know ahead of time the specific proteins to be targeted. Because our approach identifies relative differences in signal intensity between neoplastic and normal phenotypes, and can detect very small proteins (< 7,000 Daltons), it has the ability to describe a broad range of protein expression patterns that may typify neoplastic change.

Angiogenic growth factors: In addition to protein patterns in NAF, we will investigate two angiogenic growth factors, vascular endothelial growth factor (VEGF), and basic fibroblast growth factor (bFGF). These factors have been linked to vascularization of the growing tumor, a necessary early step in breast carcinogenesis. VEGF expression in breast tumors has been shown to correlate strongly with objective indicators of angiogenesis, such as microvessel density determined by factor VIII-related antigen (21,22). VEGF expression has also been shown to be significantly higher in breast cancer cells than in adjacent nonneoplastic tissue (23), to be increased in DCIS and invasive cancer compared to breast tissue from healthy women (24), and to be a significant prognostic factor (21,25,26). bFGF is another endothelial cell-specific mitogen associated with angiogenesis in breast cancer and other tumors. Increased levels of bFGF are found in tissue, serum or urine from breast cancer patients compared to that of healthy controls (26,27). Because VEGF and bFGF are associated with both angiogenesis and acquisition of the malignant phenotype, it is possible that changes in levels of these markers presage early stages of breast cancer development. A recent study found that bFGF but not VEGF was increased in NAF from breast cancer cases compared to controls, but the study was very small (10 cases, 14 controls), and did not provide details on the stage of the tumors, nor did it include patients with pre-invasive lesions (28). Our study will allow more thorough assessment of these factors, and will allow correlation between protein expression patterns and these specific growth factors.

HYPOTHESES

We propose to examine the above biomarkers in NAF from women in the following three groups (which will be matched on age and race):

(1) 40 women with normal breasts and no significant family history of breast cancer.

(2) 40 women with ductal carcinoma in-situ (DCIS).

(3) 40 women with newly diagnosed early-stage invasive breast cancer

The hypotheses to be tested are as follows:

H1: Protein expression profiles, bFGF and VEGF in **NAF** will discriminate among normal women without major breast cancer risk factors, women with DCIS, and women with early-stage invasive breast cancer. For the two growth factors, levels in NAF will increase with increasing neoplastic progression.

H2: Protein expression profiles, bFGF and VEGF in **serum** will discriminate among normal women without major breast cancer risk factors, women with DCIS, and women with early-stage invasive breast cancer. For the two growth factors, levels in NAF will increase with increasing neoplastic progression.

EXPERIMENTAL DESIGN AND METHODS

Recruitment of study subjects. Procedures at JHU and GUMC will be described separately. Although women with newly diagnosed breast abnormalities are potentially in an emotionally difficult frame of mind, we have had excellent participation in our prior studies of NAF due to cooperation with the oncologists and surgeons who treat these subjects.

JHU Recruitment

Women will be recruited from the Johns Hopkins Radiology Department. Women with normal mammograms will be recruited to Group 1. Women scheduled for a breast aspiration or biopsy will be recruited at potential subjects for Groups 2 and 3. After the Research Assistant administers informed consent to interested women, she will collect a 10cc blood sample using a standard phlebotomy protocol, and will administer an epidemiologic questionnaire. Blood samples will be kept at room temperature and transported to the lab for centrifugation and storage. Eligible participants will undergo breast nipple aspiration of both breasts following their mammogram (Group 1) or at least 5 days prior to the scheduled breast aspiration or biopsy (Groups 2 and 3). Women whose pathology reveals DCIS or early (Stage I) invasive breast cancer will be retained for Groups 2 and 3, respectively. Dr. Kathy Helzlsouer, a breast cancer oncologist and epidemiologist, will facilitate recruitment of subjects at JHU.

GUMC Recruitment

We will recruit healthy women with normal breast cancer risk (Group 1) from the Comprehensive Breast Clinic (CBC) which includes a large number of annual visits from *healthy women* with a wide range of risk profiles, including women with a normal risk of breast cancer. Women with DCIS (Group 2) will be accrued from the Breast Cancer Consultation Group, a multi-disciplinary clinic for newly diagnosed breast cancer patents, and the Department of Surgery. Women with newly diagnosed stage I breast

cancer *prior to definitive surgery* (Group 3) will be accrued from the Breast Cancer Consultation Group and the Department of Surgery of Georgetown University Medical Center. Dr. Marie Pennanen is a breast cancer surgeon who sees patients in all of these clinics and has collaborated on pilot studies of NAF; she will facilitate recruitment of subjects at GUMC.

Nipple aspiration procedure. The protocol for obtaining NAF is well established and has been refined over more than 30 years. A modified breast pump will be used. The modified pump is comprised of a highly polished and smoothed glass or plastic cup attached (via a Luer-lok) to a standard 15 ml syringe. This pump is more efficient at obtaining NAF than conventional breast milk pumps. The woman will first massage her breasts for several minutes from the base toward the nipple to increase movement of fluid toward the duct openings. A warm compress may also be placed over the breast for several minutes prior to the breast massage. After cleansing the nipple with alcohol, the cup is placed over the nipple, and the woman compresses the base of her breast with both hands while the plunger of the syringe is withdrawn to 10 ml and held for 8-10 seconds. With this technique, NAF can usually be obtained from 50-70% of premenopausal women, with steadily decreasing percentages as women age beyond menopause. The device will first be applied to the woman's forearm to give her a feeling for the pressure that the pump will exert. Several attempts of 8-10 seconds each will be made to obtain fluid. Both breasts will be aspirated. In Dr. Trock's pilot studies, and in many studies over 30 years at other institutions this procedure is well tolerated with little or no discomfort. The woman can stop the procedure at any time if it does become too uncomfortable. NAF samples collected in a capillary tube, and then diluted directly into 100 microliters of PBS and stored at -80° C.

SELDI protein expression analysis. The SELDI instrument characterizes the mixture of bound proteins by laser desorption, coupled to traditional mass spectrometry (MS) time-of-flight (TOF) analysis. It should be noted that protein separation prior to MS analysis is performed by the SELDI apparatus directly on the chip prior to MS analysis. Proteins will desorb and ionize on surface of the chip in response to the application of laser energy. Once ionized, they will release off of the chip and the ion will travel down a vacuum tube and strike a sensitive detector plate that senses ionic charges. Depending on their size, the ions will take differing amounts of time for this distance to be traveled. Larger proteins will take longer to travel, and smaller proteins will take shorter.

The peak identification program used by the SELDI machine utilizes the inherent noise generated by analysis of any sample as a reference for the potential proteins detected. Each sample will have its own background electronic "noise" without the input of any sample. This noise is calculated based on the average intensity of the mass area seen in the range spanning 250,000 to 300,000 Daltons. This region of the chromatogram will almost never contain protein signatures since the laser intensity is too small to release proteins of this size. In addition, proteins of this size are extremely inefficient in ionizing and releasing off of the bait surface. The software analysis package quantifies the background signal represented by this reference noise and identifies only those proteins whose signature intensity is some integer multiple of the noise (e.g. will identify only proteins with signal four times greater than the noise). The user can vary this multiple to change the breadth of proteins captured by the analysis. The chromatographic mass maps generated by the software can then be referred to standard libraries of known proteins at specific molecular weight. *If a particular protein peak identified in a sample does not correspond to any known protein, comparison among samples can still determine whether the particular peak occurs more or less frequently for a given patient category. Thus, even an unknown protein can be indicative of a protein signature correlated with a disease state.*

Data generated by the SELDI software are directly exported into a Microsoft Excel spreadsheet, providing absolute values for relative intensities of specific molecular weight ranges, ratios of intensity

between specified peaks, and coefficients of variation (CV) and other measures of the variability of these values, i.e. standard deviations, and mass accuracy (equivalent to the standard error of the mean intensity). These data are readily compatible with the study Microsoft ACCESS database, and standard statistical analysis software such as SAS.

In optimization studies performed by Dr. Petricoin in the initial year of this grant, the hydrophobic (aliphatic) reverse phase chip (H4 Protein Chip™, Ciphergen, Palo Alto, CA.) provided the broadest and most consistent protein peak identification in samples from breast tissue, NAF and serum. This chip will be used for the proteomic analyses in the study samples. The chip is pretreated with 1 microliter of acetonitrile (Sigma, St.Louis, MO). Shortly before the acetonitrile completely evaporates the sample is applied to the bait surface (5 microliter aliquot of the diluted NAF or serum). The analyte is then allowed to concentrate by air-drying followed by washing two times for 5 minutes in 1X PBS. 0.3 microliters of a saturated solution of 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid, Sigma, St. Louis, MO), the energy absorbing molecule of choice, is applied to the washed surface of the chip and allowed to crystallize. The chip is then subjected to laser desorption and TOF analysis. Similar approaches are used with other chips, although the reagents and washing conditions vary. *All SELDI assays will be conducted blinded to the histologic class of the samples.*

ELISA Assays of bFGF and VEGF. Samples will be assayed in triplicate according to the instructions of the manufacturer (R&D Systems, Inc., Minneapolis MN), using the same methods we successfully used in our pilot study of bFGF in NAF, and which are similar to the methods used by Relf and colleagues (26). 25 microliter samples diluted in PBS buffer will be incubated at 4°C on 96 well microtiter plates coated with monoclonal antibody to human bFGF or VEGF. After washing to remove unbound protein, an enzyme-linked polyclonal antibody for the specific cytokine will added. Colorimetric analysis at 450 nm will determine the amount of cytokine bound to antibody. Two positive and two negative controls will be included in each plate. The assay for bFGF has a sensitivity of < 0.3 pg/ml, and the assay for VEGF has a sensitivity of < 9 pg/ml, both of which are well below the levels expected based on bFGF and VEGF levels reported by Liu (100 – 7000 pg/ml) (28).

Statistical Analysis. Proteomic analyses will take two approaches. First, we will identify specific protein peaks that are present at much greater intensity in one histologic component (for example, invasive breast cancer) than in the others. Within each histologic category, the intensity of such a differentially expressed peak will be compared to the intensity of a peak that does not appear to vary greatly across histologies, with this comparison expressed as a ratio. The SELDI software allows you to choose any two peaks to express as a ratio. This ratio will be averaged for the particular histologic component (e.g., invasive) over all patients. Similarly, the ratio will be averaged across all patients for each of the other two histologic components (normal and DCIS). Then, paired t-tests can be used to make pairwise comparisons of these averages between histologic categories (e.g. invasive vs. DCIS). Analysis of variance with blocking on the matched triple (normal, DCIS, invasive) can be performed to compare the average ratio of intensities for a candidate protein peak across all three histologies simultaneously, and also allows incorporation of potential confounding factors (since all three histologies are matched within each patient, we don't anticipate major confounding).

The second analytic approach will focus on multiple peaks that appear to be differentially expressed across histologic categories, to identify *patterns* of altered protein expression. The SELDI software allows you to screen for peaks that appear to be frequently expressed at a specified multiple of the intensity of a designated peak that doesn't appear to differ across histologies (for example, if a peak at 7800 Dalton appears to be present at relatively similar intensities across histologic categories, the software can identify all peaks whose intensity is a specified multiple, say threefold, of the peak at 7800

Dalton). Then, the proportion of patients who exhibit increased expression of these peaks can be determined within each histologic category (e.g. 80% of patients exhibit increased expression of the candidate peaks in their invasive component). These proportions are then compared pairwise between histologic categories using a McNemar chi-square for matched pairs. All three histologic groupings can also be compared simultaneously using conditional logistic regression for a 1 to 2 matched design (29).

It should be noted that in both of the above types of analysis, histologies can be combined, i.e. normal and DCIS can be combined to compare with invasive. Furthermore, peaks whose intensity appears to be *under expressed* relative to a fairly constant peak can also be examined.

For analyses of bFGF and VEGF we will compare mean levels of these markers among each of the three groups. Paired t-tests (or nonparametric alternatives if the distribution is not Gaussian) will compare growth factor levels in a pairwise fashion (i.e. Group 1 vs 2, Group 1 vs 3, Group 2 vs 3). Analysis of covariance with blocking on the matched triple (i.e. each matched set of Groups 1, 2 and 3) will be used to compare all three groups simultaneously, and will allow adjustment for potential confounding factors such as reproductive factors, family history, body mass index, or phase of the menstrual cycle.

Sample size and power. Power will be based on the more stringent analyses, i.e. the proteomic studies. The power calculation for the first type of analysis described above will be based on the paired t-test. Because we have no basis for predicting *a priori* the absolute difference in mean ratio of intensities that will be a relevant discriminant, we can base our power calculation on the size of the mean difference relative to the standard deviation. Our preliminary data show that the coefficients of variation (CV) for the ratios of intensity range from about 4% to 70%. That means that at the highest level of variability observed in the preliminary data, the standard deviation is 70% of individual ratio means. To be conservative we will base our sample size on higher levels of variability, i.e standard deviations that are 100% and 150% of the individual means. We will also conservatively estimate that the correlation between ratios in the different histologic categories is very low, rho = 0.10. With 40 patients, each representing all three histologies, the power is > 0.99 to detect differences in mean ratio of intensity between histologies, when the standard deviation is 100% of the mean difference in ratio of intensities. Even if the standard deviation is 150% of the mean difference, the power = 0.88 (30). Power is actually likely to be higher than that given here, since we assumed a low correlation between histologic classes, so power will be higher if the correlation is higher. Thus, we will have excellent power to detect meaningful differences in intensity of specific peaks. It should be noted that even if the Type I error level is decreased to 0.01 or 0.02 to provide more stringent protection against multiple comparisons problems, power will still exceed 0.90 or 0.80 for standard deviations of 100% and 150% of the mean, respectively.

For the second analysis described in Section D1.6, we will compare across the three histologic classes the proportion of patients with the pattern suggesting altered expression of the candidate proteins. This proportion calculated for the invasive histologic component can be considered to be the sensitivity (Se) of the candidate protein pattern for detection of invasive cancer (i.e. the proportion of patients whose invasive cancer component exhibits the candidate pattern). For the normal and DCIS components, this proportion will be 1- specificity (1 - Sp), if we consider Sp = proportion of patients whose normal (or DCIS) component does not exhibit the candidate pattern. Therefore, our power calculation can be based on identifying a protein pattern that is associated with levels of Se and Sp that are clinically meaningful. For the approach we are proposing for early detection to be an improvement over current screening methods we would want sensitivity to be $\geq$ 80% (since mammography has sensitivity of 75% - 90%). Specificity is not routinely estimated for mammography since woman with a normal mammogram do not have a biopsy to provide a "gold standard" for comparison. However, it is unlikely that our approach

would be clinically useful if specificity was lower than 70%. For a Se = 80% and 1 - Sp = 30% (i.e. Specificity = 70%), the power = 0.88 with 40 pairs (i.e. comparing invasive to DCIS) (30). If Se > 80%, or Sp > 70% (i.e. if the protein pattern has even better discriminatory ability), power will be even higher. Thus, if we take Se = 80% and Sp = 70% as the minimum levels of sensitivity and specificity that demonstrate acceptable discriminatory ability for a particular protein signature (pattern of proteins with altered expression), power will be more than adequate to detect such differences in protein pattern between histologic components.

HUMAN SUBJECTS ISSUES

Risks. The pressure exerted by the pump is generally regarded as being only mildly uncomfortable (most women describe it as less uncomfortable than a mammogram). There is a very small chance that a bruise or minor abrasion may develop where the pump is placed on the breast. The procedure for obtaining NAF will not have any impact on the results of any subsequent breast biopsy or surgery, or on the healing process. If a blood sample is obtained, mild pain or bruising, lightheadedness or infection may result from the needle stick and usually improves without any medical intervention.

Benefits. Subjects will not directly benefit from providing these specimens, although future generations may benefit from increased medical knowledge about breast cancer. Because these samples will be used for research purposes only, subjects will not receive specific information on the results of any research tests performed on their samples.

Payment for Participation. Subjects will not be paid for providing specimens.

Compensation for injury. The Johns Hopkins University, the Johns Hopkins Hospital, the Johns Hopkins Bayview Medical Center and the federal government do not have a program to pay for the medical care of subjects who are hurt or experience adverse events associated with participating in this study. Subjects will be responsible for payment of any treatment or hospitalization required if they are injured. This is the standard policy of Johns Hopkins for most non-treatment studies.

Commercial Issues. Subjects will not be compensated for any products developed from this research.

Modification of the Protocol. Any changes to the procedures or activities described herein will be submitted as amendments to the WIRB and to the IRB from GUMC for review and approval prior to implementation.

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Certificate of Approval

THE FOLLOWING WERE APPROVED:

INVESTIGATOR: Bruce J. Trock, Ph.D.
Johns Hopkins University
School of Medicine
Jefferson Street Building, Room 149
600 North Wolfe Street
Baltimore, MD 21287-8915 USA

BOARD ACTION DATED: 01-25-2002
PANEL: 8
APPROVAL EXPIRES: 01-25-2003
STUDY NR: 1035603
WIRB PRO NR: 20020089
INVEST NR: 67483

SPONSOR: National Cancer Institute
PROTOCOL NR: U01-CA85082-01
AMD. PRO. NR:
TITLE:
Detecting Breast Cancer Protein Signatures in Body Fluids

APPROVAL INCLUDES:
Initiation of Investigations Under This Research Grant
Recruitment of Human Research Subjects
Protocol
Consent Form - As Modified by WIRB
Investigator

WIRB APPROVAL IS GRANTED SUBJECT TO:
(See Back of this Certificate)

ALL WIRB APPROVED INVESTIGATORS MUST COMPLY WITH THE FOLLOWING:

1. Conduct the research as required by the Protocol;
2. Use only the Consent Form bearing the WIRB "APPROVED" stamp;
3. Provide non-English speaking subjects with a certified translation of the approved Consent Form in the subject's first language. The translated version must be approved by WIRB;
4. Obtain pre-approval from WIRB of any changes in the research activity (except when necessary to protect human subjects; 21 CFR § 56.108(a)(3)); immediately report to WIRB any such emergency changes for the protection of human subjects;
5. Report to WIRB the death, hospitalization, or serious illness of any study subject;
6. Promptly report to WIRB any new information that may adversely affect the safety of the subjects or the conduct of the trial;
7. Provide reports to WIRB concerning the progress of the research, when requested;
8. Obtain pre-approval of study advertisements from WIRB before use;
9. Conduct the informed consent process without coercion or undue influence, and provide the potential subject sufficient opportunity to consider whether or not to participate.

Federal regulations require that WIRB conduct continuing review of approved research. You will receive Continuing Review Report forms from WIRB. These reports must be returned even though your study may not have started.

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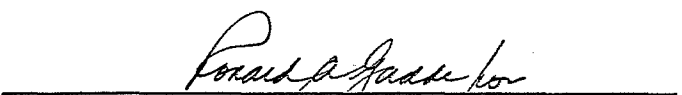
INSTITUTION: Johns Hopkins University **CONTACT:** Barbara Starklauf

IF YOU HAVE ANY QUESTIONS, CONTACT WIRB AT 1-800-562-4789

This is to certify that the information contained herein is true and correct as reflected in the records of the Western Institutional Review Board (WIRB). WE CERTIFY THAT WIRB IS IN FULL COMPLIANCE WITH GOOD CLINICAL PRACTICES AS DEFINED UNDER THE U.S. FOOD AND DRUG ADMINISTRATION (FDA) REGULATIONS AND THE INTERNATIONAL CONFERENCE ON HARMONISATION (ICH) GUIDELINES.



William C. Jacobs, Chairman

FEB 0 6 2002

(Date)

JOHNS HOPKINS
MEDICINE

General Clinical Research Center

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June 13, 2002

Bruce Trock, Ph.D.
Associate Professor of Urology, Epidemiology, Oncology
149 Jefferson Bldg,
Johns Hopkins Medical Institutions

RE: "Detecting Breast Cancer Protein Signature in Body Fluids."

Dear Dr. Trock;

Thank you for requesting the use of the General Clinical Research Center to support your research project. Your study was approved as a Category "A" study on June 5, 2002.

We would like you to respond to a few points:

1. The Committee suggests that you talk with the Breast Cancer Group.
2. Will you be doing genetic testing?
3. One concern was that the proposed statistical analysis may not be adequate to deal with comparisons among the three groups.

Proof of human subjects training certification will be required for all key personnel listed on your grant prior to use of GCRC resources. The GCRC cannot support research projects with pending, lapsed, or terminated IRB approval.

The following contacts will be helpful to start your study. Nursing inservices will be required for all units involved.

Nurse Manager, Phyllis Pentz 5-2763
Visit Registration, GCRC Administration, 4-2717
Account Set-up, Mary Kirkendall, 4-2717

Please remember to provide a copy of amendments to the protocol and / or consent forms to GCRC Administration, Carnegie 446 if and as they occur. All changes to Co-Investigators and / or Collaborators need to be brought to the attention of the JCCI and their name added to the consent.

NCRR-Supported Research

We ask that you remember to include the GCRC citation "Supported by the Johns Hopkins University School of Medicine General Clinical Research Center's grant # M01-RR00052, from the National Center for Research Resources/NIH" when publishing articles related to this research.

Sincerely,

Michael J. Klag, M.D., MPH
Vice Dean for Clinical Investigation
Chairman, GCRC Advisory Committee

Simeon Margolis, M.D., Ph.D.
Professor, Endocrinology
Adult Protocol Review
Subcommittee

LIST OF ABBREVIATIONS AND ACRONYMS

DCIS	ductal carcinoma in-situ
NAF	nipple aspirate fluid
FGF-2	basic fibroblast growth factor
VEGF	vascular endothelial growth factor
JHU	Johns Hopkins University
SELDI	surface enhanced laser desorption and ionization spectroscopy
OHRP	Office for Human Research Protection
WIRB	Western Institutional Review Board
GCRC	General Clinical Research Center
ELISA	enzyme-linked immunosorbent assay

Meeting abstracts during reporting period: None in connection with this project

Publications during reporting period: None in connection with this project

Manuscripts in preparation: None in connection with this project

Personnel receiving pay from this negotiated effort:

Bruce Trock, Ph.D.
Dorraya El-Ashry, Ph.D.
Michelle Brotzmann, MPH
Joseph Alexander